Broadly expressed repressors integrate patterning across orthogonal axes in embryos

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The role of spatially localized repressors in supporting embryonic patterning is well appreciated, but, alternatively, the role ubiquitously expressed repressors play in this process is not well understood. We investigated the function of two broadly expressed repressors, Runt (Run) and Suppressor of Hairless [Su(H)], in patterning the *Drosophila* embryo. Previous studies have shown that Run and Su(H) regulate gene expression along anterior-posterior (AP) or dorsal-ventral (DV) axes, respectively, by spatially limiting activator action, but here we characterize a different role. Our data show that broadly expressed repressors silence particular enhancers within *cis*-regulatory systems, blocking their expression throughout the embryo fully but transiently, and, in this manner, regulate spatiotemporal outputs along both axes. Our results suggest that Run and Su(H) regulate the temporal action of enhancers and are not dedicated regulators of one axis but, instead, act coordinately to pattern both axes, AP and DV.

embryonic patterning | transcriptional repressor | enhancers | Su(H) | Runt

Patterning of embryos is accomplished through the combinatorial action of transcription factors, many having spatially localized expression domains, but how broadly expressed, often ubiquitous, factors support gene expression is less well understood. In *Drosophila* embryos, the maternally deposited transcription factors Bicoid and Dorsal are present in gradients oriented along the anterior-posterior (AP) and dorsal-ventral (DV) axes, respectively (1, 2). These transcription factor gradients act as concentration-dependent inputs that pattern each axis, supporting their classification as morphogens. Patterning results from integration of positive and negative input from these and other spatially localized transcriptional activators and repressors to support gene expression within distinct domains along the two orthogonal body axes (1, 3). However, more recent studies have determined that broadly expressed, pioneering activators also play a role. The maternally deposited activator Zelda impacts patterning globally throughout the embryo, influencing gene expression along AP and DV axes (2). Zelda is able to augment the ability of Bicoid and Dorsal, and likely other transcription factors as well, to support activation of gene expression, in part, by increasing their access to DNA (4). Less is known regarding the mechanism of action of ubiquitous, or broadly expressed, repressors.

Broadly expressed repressors Runt (Run) and Suppressor of Hairless [Su(H)] have been linked to patterning the AP and DV axes, respectively (5–7). Run repressor activity influences Bicoid-mediated activation of gap genes by helping to establish posterior boundaries of genes expressed more anteriorly along the AP axis (6). Alternatively, Su(H) acts as a repressor to define boundaries of genes along the DV axis. Whereas Run sets positional boundaries in a particular domain of the early embryo (6), Su(H) acts broadly to counterbalance Dorsal-mediated activation along the DV axis (7). Su(H) and Dorsal binding sites exhibit overlap, and, moreover, increasing or decreasing the ratio of Su(H) to Dorsal binding sites when placed in tandem influences gene boundary positions across the DV axis, suggesting that these factors function antagonistically. These particular studies provided important insight into the roles for Run and Su(H) and also suggested that these transcription factors provide dedicated input to AP or DV axis patterning, respectively.

However, our data here show that Run and Su(H) have more widespread roles in patterning the embryo, as they act to transiently silence the activity of particular enhancers throughout the entire embryo. This leads to delayed action of select enhancers within *cis*-regulatory systems to regulate gene expression spatiotemporal dynamics across both axes, AP and DV.

Results and Discussion

Similarity of Su(H) DNA Binding Site Consensus to AP Enhancer-Associated Motif and Run Site. In a previous study, we conducted ChIP experiments coupled with high-throughput sequencing (ChIP-seq) to examine the in vivo binding occupancy of Su(H) transcription factor to DNA within *Drosophila* embryos (7). We noticed that the Su(H) binding site, derived in vivo from ChIP-seq–identified peaks (Fig. 1*A*) or in vitro studies (Fig. 1*B*, *Top*), overlaps with the MEME motif-derived site identified by Chen et al. (6) as an overrepresented sequence present in AP enhancers, AYCCRCA (Fig. 1*B*, *Bottom*). In this previous study, similarity between the Run DNA binding site sequence WAACCRCAR (JASPAR) and this AP enhancer motif led to identification of an earlier role for Run in antagonizing Bicoid-mediated activation (6) (Fig. 1*B*). We hypothesized that Su(H) might also support a role in regulating patterning along the AP axis, so we began by closely examining the expression pattern of Su(H) compared with that of Run in the early embryo.

Even Though Su(H) Is Broadly Expressed, Mutant Embryos Exhibit AP Patterning Defects That Perdure. Run and Su(H) transcription factors exhibit dynamic expression patterns, which, at times, include patterns that are localized broadly throughout the embryo

Significance

Molecular mechanisms that establish the body plans of multicellular animals are not fully understood. This study was focused on two key transcription factors, Runt (Run) and Suppressor of Hairless [Su(H)], that act on particular enhancers within *cis*-regulatory systems. We showed these factors impact anterior-posterior and dorsal-ventral patterning by supporting enhancers to coordinate their action. These repressors can simultaneously regulate patterning across orthogonal axes and act as a counterbalance to the action of ubiquitous activators such as Zelda. Shared use of broad transcription factors, like Su(H)/Run, across axes may help to integrate patterning throughout the embryo and support robust development. Roles for broadly expressed repressors in the regulation of enhancer timing is likely a conserved mechanism of action in higher animals.

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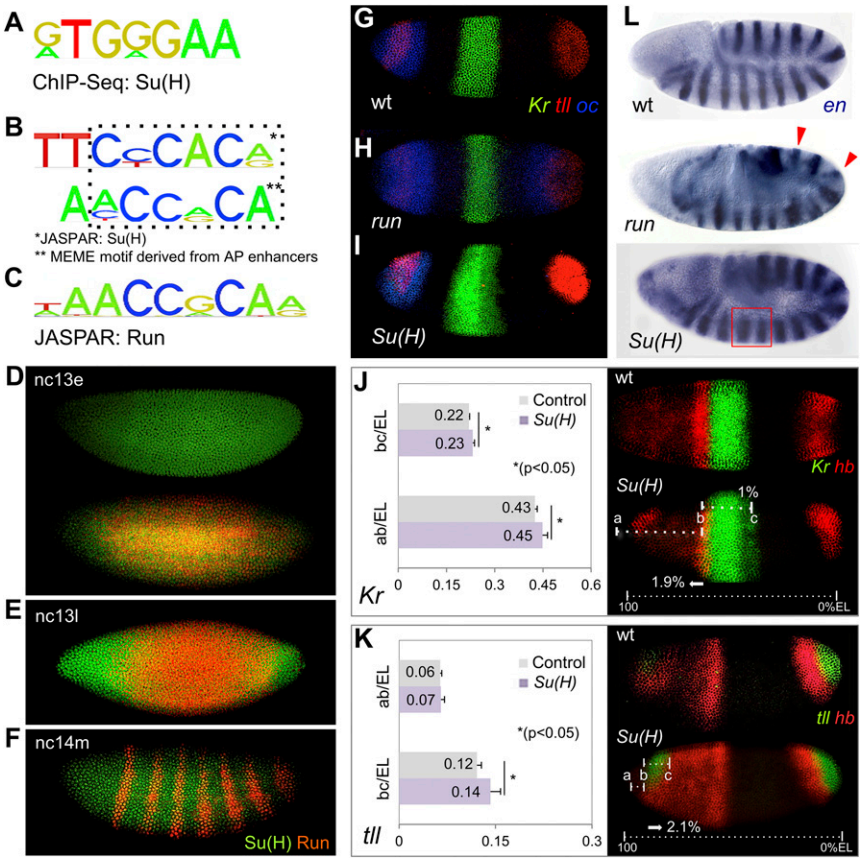

Fig. 1. Su(H) binding site and mutant phenotypes suggest a role for Su(H) in AP patterning. In this and all subsequent figures, *Drosophila* embryo images are depicted with anterior to the left and dorsal up unless otherwise noted. (*A*–*C*) Comparison of Su(H) DNA binding consensus site derived from ChIP-seq (*A*; 24% of called peaks compared with 6% background) (7), *Drosophila* Su(H) motif from JASPAR (*B*, *Top*; reverse complement relative to *A*), motif overrepresented within AP enhancers (*B*, *Bottom*) (6), and *Drosophila* Run consensus binding site (*C*) (6). (*D*–*F*) Anti-Su(H) (green) and anti-Run (red) protein staining of embryos at early stage nc 13 (nc13e; *D*), late nc 13 (nc13l; *E*), and mid-nc 14 (nc14m; *F*). (*G*–*I*) FISH using riboprobes to detect *Kr* (green), *tll* (red), and *oc* (blue) transcripts in WT embryos (*G*) as well as in *run* (*H*) and *Su(H)* (*I*) mutant embryos. (*J* and *K*) Ratio of *Kr* and *tll* transcript expression domains relative to total EL. Embryos processed by FISH using *Kr*, *hb*, and/or *tll* riboprobes to detect transcripts in WT and *Su(H)* mutants at early nc 14. The anterior boundaries of the central *Kr* (*J*) or anterior *tll* (*K*) domains are marked as "ab," whereas "bc" demarcates the central, dorsal, *Kr* domain width (*J*), or anterior *tll* domain length (*K*). (*L*) *en* transcript expression in germband-elongated embryos of WT, *run* mutant, or *Su(H)* mutant genetic backgrounds. Red arrowheads mark odd *en* stripes in *run*$^-$ embryos, whereas a red box indicates the *en* interstripe distance in *Su(H)* mutants.

(8, 9). Previous studies had shown that Run is expressed in the trunk of *Drosophila* embryos but excluded from the terminal ends, early during nuclear cycle (nc) 13 (Fig. 1 *D* and *E*) (9), whereas, later, at nc 14, the pattern refines into a pair-rule expression pattern composed of seven stripes oriented along the AP axis (Fig. 1*F*). Most Run studies had focused on its role as regulator of pair-rule expression (10), but it was shown more recently that broadly expressed Run, at early stages, functions as a repressor of gap genes to position the boundary of genes expressed more anteriorly along the AP axis (6). In contrast, the Su(H) protein is broadly expressed in the early embryo (Fig. 1 *D*–*F*), even though lower levels of Su(H) are present at the anterior, specifically at nc 13 (Fig. 1*D*, *Top*). Su(H) repression activity regulates genes along the DV axis, except in ventrolateral and, possibly, also in ventral regions where input from Notch signaling pathway activation switches Su(H) from repressor to activator (5). It was not clear at first how these broadly expressed repressors could impact patterning spatially across both axes, which are orthogonal. Nevertheless, we investigated mutants for patterning phenotypes.

Gap gene expression along the AP axis was examined in embryos derived from *Su(H)* mutant germline clone females and compared with phenotypes described previously for zygotic *runt* (*run*) mutants. Run had been shown to regulate the expression of a subset of genes expressed along the AP axis (1). For example, in *run* mutants, the expression domain of the *ocelliless* (*oc*) gene is expanded at the posterior end, whereas the domain of *Krüppel* (*Kr*) expression is decreased in width (Fig. 1*H* vs. Fig. 1*G*). However, not all patterns are changed, as anterior *tailless* (*tll*) expression remains essentially unchanged in *run* mutants. We analyzed *Su(H)* mutant embryos lacking maternal and zygotic gene function obtained from female germline clones and found that they also exhibit AP patterning defects in addition to the DV patterning phenotypes described previously (5, 7). In *Su(H)* mutant embryos, *oc* expression is unchanged; however, the central *Kr* and anterior *tll* expression domains are expanded (Fig. 1*I* vs. Fig. 1*G*). When these expression domains were measured and normalized to embryo length, we found evidence that the boundary positions of *Kr* and *tll* were expanded posteriorly in the *Su(H)* mutants (Fig. 1*J*,*K*). Although *run* and *Su(H)* mutant embryo phenotypes are different, these results suggest that both genes play a role in patterning the AP axis.

Furthermore, these mutants exhibit phenotypes affecting expression of *engrailed* (*en*) at later stages (Fig. 1*L* and Fig. S1). In embryos undergoing germband elongation, En transcription factor, a segment polarity factor, is expressed in 14 stripes along the length of embryos and controls segmentation (11). We found that *en* phenotypes are exhibited by *Su(H)* mutants, as shown previously for *run* mutants (12), but the phenotypes differ. En stripes are broadened in *Su(H)* mutants (Fig. 1*L*), and the interstripe distance is increased upon *Su(H)* ectopic expression (Fig. S1*C* vs. Fig. S1 *A* and *B*). These results show that Run and Su(H) mutations exhibit lasting effects on AP patterning.

Su(H) Regulates All Three Types of Bicoid-Bound AP Enhancers Compared with a More Targeted Role for Run. As embryos derived from *Su(H)* germline clone mutant females exhibit alterations in AP patterning (Fig. 1), we examined whether Su(H) regulates AP enhancers. The Bicoid gradient supports gene expression along the AP axis (Fig. 2*A*), but posterior boundaries of targets, which fall into a broad domain within the embryo, are likely specified by other factors, possibly Su(H) (Fig. 2*B*). In a study by Chen et al., 66 enhancers were characterized that support expression along the AP axis, and many were identified based on ChIP-defined occupancy of Bicoid transcription factor to these DNA sequences in vivo (6). This collection of enhancers

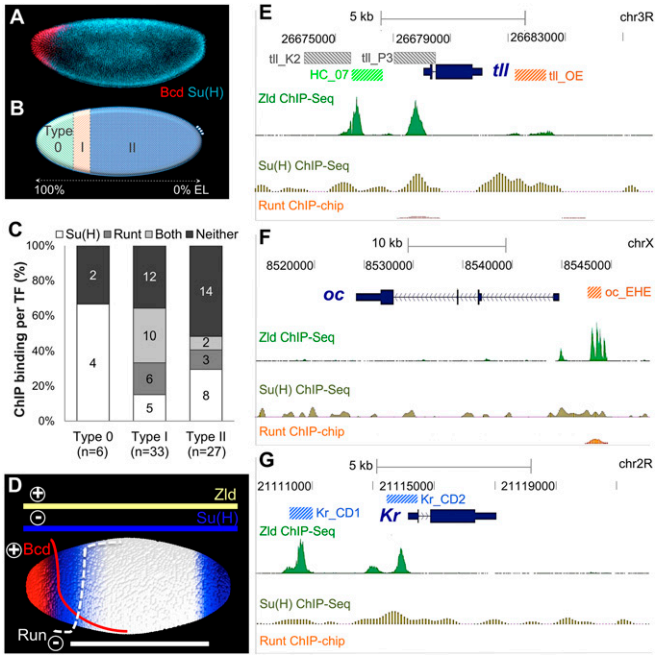

Fig. 2. Transcription factors binding to three different enhancer types that drive expression along the AP axis. (*A*) Su(H) (cyan) and Bicoid (Bcd; red) protein expression in early nc 14 embryos. (*B*) Schematics of three Bcd-defined enhancer types, type 0 (green), type I (orange), and type II (light blue), classified by the positions of their Bcd-dependent posterior boundaries based on Chen et al. (6). Left end of schematic represents anterior tip of the embryo (100% EL). (*C*) A total of 66 Bcd-dependent enhancers derived from Chen et al. (6) were classified based on whether endogenous sequences exhibit Su(H) and/or Run ChIP binding or a lack thereof ("Neither"). (*D*) Representation of Zld, Su(H), Bcd, and Run expression patterns within a schematic of an early embryo. Colored lines represent regions of expression for each of these factors. "+" defines input by activators Zld and Bcd, and "−" defines input by repressors Su(H) and Run. (*E–G*) ChIP binding data for Zelda (13), Su(H) (7), and Run (14) showing occupancy of these factors at three loci: *tll* (*E*), *oc* (*F*), and *Kr* (*G*). Location for previous characterized enhancers active in the early embryo are shown and colored if associated with type 0 (green), type I (orange), or type II (blue) AP enhancers (*B*) or none (gray).

was classified into three groups based on the position of posterior boundaries of expression supported by these reporter constructs: six type 0 patterns [boundaries in 100–85% egg length (EL) domain]; 33 type I patterns (boundaries in 85–75% EL); and 27 type II patterns (boundaries in 75–0% EL domain; Fig. 2*B*) (6). Zelda is a ubiquitous activator that binds most *cis*-regulatory sequences in the early *Drosophila* embryo (13). Cooccupancy of Zelda with other transcription factors, including Bicoid and Run (14), is often associated with many of these AP enhancer sequences. In a previous study, we identified occupancy of Su(H) to many enhancers, supporting expression along the DV axis (7), but, in the course of this work, we also noticed binding to AP enhancers as well. Comparing binding of Zelda, Su(H), and Run shows that these factors often, but not always, exhibit cooccupancy on the DNA in regions shown to act as enhancers (Fig. 2 *E–G*).

We investigated whether ChIP binding could be used to infer roles for Su(H) and Run in regulating enhancer activity. Run has been shown to modulate type I patterns. The sequence of the Run DNA binding site is enriched within type I enhancers, and *run* mutants exhibit alterations of these patterns (6). As would be expected, Run ChIP-defined binding is enriched in enhancers supporting type I patterns, but we also found that it is associated with enhancers of type II patterns (Fig. 2*C*). In contrast, the ChIP-defined binding of Su(H) is broadly associated with enhancer sequences representing all three classes (i.e., types 0, I, and II; Fig. 2*C*). The broad occupancy of Su(H) to AP enhancers of types 0, I, and II classes and their wide expression range on the AP axis (Fig. 2 *A* and *B*) suggested that this factor may play an expanded role in patterning the embryo.

We hypothesized that repressors Su(H) and Run both regulate patterning along the AP axis by binding to the AP enhancers to counterbalance activation by Bicoid and Zelda (Fig. 2*D*). To test this idea, the effect of ectopic Su(H) or Run on expression of lacZ reporters supporting type 0, I, or II patterns was examined (Fig. 3). Ectopic expression was accomplished by using heat-shock expression constructs (12, 15). Three type 0 enhancers were assayed, and all exhibited repression of anterior patterns by ectopic Su(H); in contrast, ectopic Run had no effect on their expression (Fig. 3 *A* and *B*). Five enhancers of type I were assayed (Fig. 3*C*). Four were repressed by Run, including one that was additionally repressed by Su(H) (i.e., HC_35; Fig. 3*D* and Fig. S2*A*). Six enhancers of type II were assayed (Fig. 3*E*): two were repressed by Su(H) but not by Run (Fig. 3*F*, HC_09, and Fig. 4F, hb_shadow), two were repressed by Su(H) and Run (Fig. 4*G*, hb_stripe), and three were not repressed by either (Fig. 3*F*, gt23, Fig. 4*E*, hb_P2, and Fig. S2*B*, eve2). These results suggest that (*i*) Su(H) plays a major role in regulating type 0 patterns, whereas Run has marginal, if any, effect; (*ii*) Run plays a major role in regulating type I patterns, as described previously by Chen et al. (6), but Su(H) also can support this role; and (*iii*) Su(H) and Run both can regulate type II patterns and their roles in regulation of this particular class are variable,

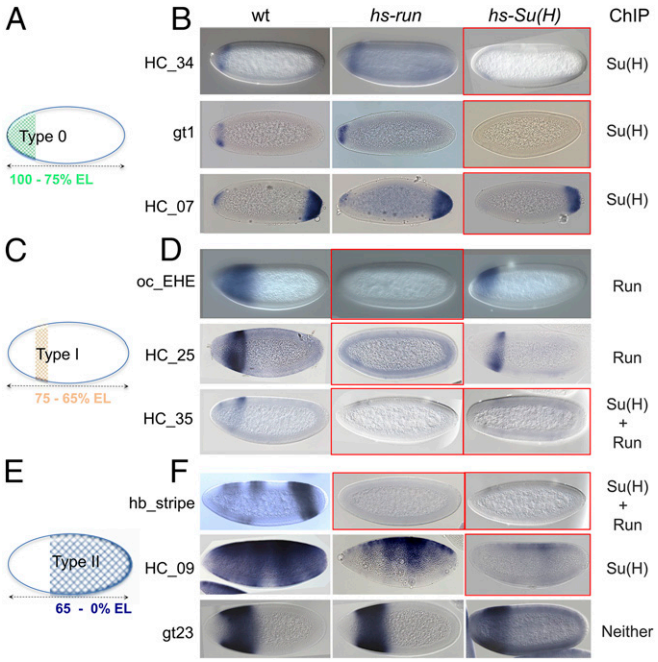

Fig. 3. Ectopic expression of Su(H) or Runt has variable effects on AP enhancers. (*A*, *C*, and *E*) Schematic of domains of expression for gap genes expressed along the AP axis of *Drosophila* embryos that fall into the type 0 (*A*), type I (*C*), or type II (*E*) enhancer categories. (*B*, *D*, and *F*) Embryos containing the indicated enhancer constructs of type 0 (*B*), type I (*D*), or type II (*F*) classification within three different genetic backgrounds, WT embryos, *hs-run*, and *hs-Su(H)*, were equivalently heat-shocked, and *lacZ* reporter expression examined by in situ hybridization. Embryos outlined in red from experiments showing loss of expression associated with heat shock. ChIP-defined binding to these enhancers sequences (endogenous locations) as assayed for occupancy by Su(H), Run, Su(H) + Run, or neither factor is shown (*Right*).

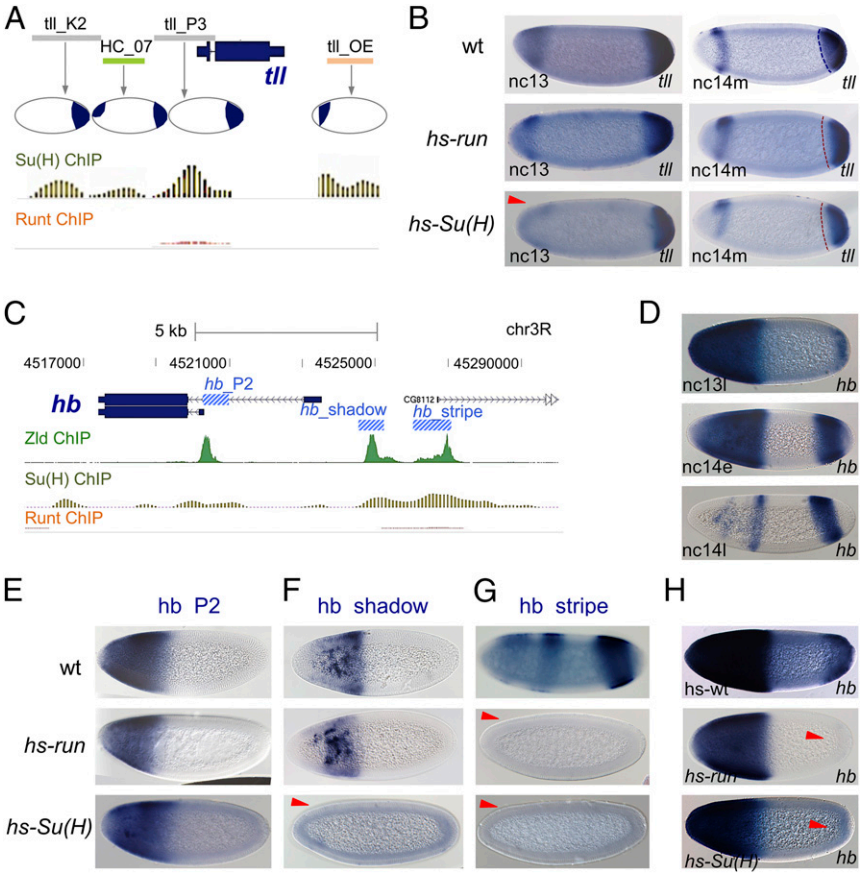

Fig. 4. Broadly expressed repressors affect gap gene patterns via impacting the timing of action for a subset of their enhancers. (*A*) Relative location of four *tll* enhancer sequences (tll_K2, HC_07, tll_P3, tll_OE) to *tll* gene as well as the pattern of expression supported by each enhancer diagramed within the embryo schematics (26) compared with Su(H) and Run ChIP-defined in vivo occupancy to these sequences. (*B*) *tll* gene expression in heat-shocked WT and *hs-run* or *hs-Su(H)* embryos at nc 13 and mid-nc 14. (C) ChIP data for Zelda, Su(H), and Run binding to the *hb* locus relative to position of three enhancers (blue boxes) (19), supporting early embryonic *hb* expression. (*D*) Endogenous *hb* expression at three stages in the early embryo detected by in situ hybridization shows that the pattern is very dynamic. (*E–H*) Expression associated with *hb* enhancer reporter constructs using *lacZ* riboprobe (*E–G*) or endogenous *hb* (*H*) in heat-shocked WT, *hs-run*, and *hs-Su(H)* embryos at mid-nc 14 (*E–G*) or at the end of nc 13 (*H*). Delayed *hb* phenotype exhibited in 12 of 15 *hs-run* and 11 of 15 *hs-Su(H)* embryos (*H*). Red arrowheads mark domains where patterns exhibit alterations.

as one, both, or neither were found to support repression. Furthermore, the ability of Su(H) or Run to completely silence expression from these reporters correlated well with binding of these factors to enhancer sequences at the endogenous loci in vivo as determined by ChIP (Fig. 3 *B*, *D*, and *F*, *Right*). The exceptions were a few cases in which Su(H) or Run was found to decrease levels of expression but were not able to abolish expression completely [Fig. S2, HC_02 partial repression by Su(H), and Fig. 4*F*, hb_shadow partial repression by Run]. In summary, these experiments showed that Su(H) can repress type 0, I, and II patterns, whereas effects by Run are limited to type I as well as some type II patterns.

Run and Su(H) Target Particular Enhancers Within the tll and hb *cis*-Regulatory Systems. To provide insight into the mechanism of action used by Run and Su(H) repressors, we investigated how these factors regulate enhancer function in their native genomic context within *cis*-regulatory systems in which multiple enhancers function coordinately (e.g., refs. 16, 17).

For example, multiple enhancers act to support embryonic expression of the gene *tll* (Fig. 4*A*). *Su(H)* mutant embryos exhibit expanded anterior *tll* expression (Fig. 1*K*), whereas it has been shown that *run* mutants exhibit expansion of anterior and posterior *tll* (18). To provide insight into the mechanism by which these repressors impact *tll* patterning, we first examined sensitivity of particular enhancers (Fig. 4*A*) to ectopic Su(H) or Run. The HC_07 *tll*-associated enhancer was repressed in anterior regions by ectopic expression of Su(H) but not Run (Fig. 3*B*, HC_07), whereas the *tll*_OE enhancer was not repressed by either factor (Fig. S2*A*). Upon heat shock-mediated ectopic expression of Run or Su(H), changes in endogenous *tll* expression were also observed (Fig. 4*B* and Fig. S3). Run and Su(H) decrease posterior *tll*, whereas only Su(H) represses anterior *tll* (Fig. 4*B*). This effect is consistent with the ChIP binding data, which showed that enhancers that support anterior *tll* expression are bound by Su(H) but not by Run (e.g., HC_07), whereas at least one enhancer that supports posterior *tll* expression is bound by both factors (e.g., tll_P3; Fig. 4*A*). These data suggest that Run and Su(H) target particular enhancers.

Furthermore, *tll* anterior expression appears delayed, rather than completely abolished, by ectopic Su(H) (Fig. S3*D* vs. Fig. S3*C*), and *tll* repression by Run at the posterior is also only transient, as the *tll* pattern appears similar to WT at late nc 14 (Fig. S3*E* vs. Fig. S3*C*). Although these results may relate to changes in the timing of enhancer action by regulating exchange from one enhancer to the next, little is known about the temporal order of action of *tll* enhancers. Therefore, we turned our focus to another gap gene, *hunchback* (*hb*), which exhibits dynamic expression (Fig. 4*D*) that is supported by the coordinated activity of three enhancers.

hb is regulated by three distinct noncoding regions, enhancers hb_P2, hb_shadow, and hb_stripe (Fig. 4*E*, *Top*) (19). Early expression is supported by the hb_P2 within a cap at the anterior 40% of embryos. The hb_shadow pattern overlaps in expression with hb_P2 but also exhibits a sharper posterior boundary than hb_P2. Finally, the hb_stripe enhancer supports expression in a stripe localized at ~40% EL (from the anterior pole) as well as in a domain at the posterior of the embryo. Several previous studies support the view that these three enhancers function together to support the dynamic *hb* gene expression pattern (Fig. 4*D*) (16, 19). We investigated how Run and Su(H) affect expression of *hb* and these three embryonic enhancers. *hs-run* expression acts to silence expression from hb_stripe, but has only a marginal effect on expression of hb_P2 and hb_shadow (Fig. 4*E*). In turn, *hs-Su(H)* expression acts to silence expression of hb_shadow in addition to hb_stripe, but has only minimal effect on expression of hb_P2

(Fig. 4*E*). Although binding of Su(H) was detected at the hb_P2 sequence (Fig. 4*C*), silencing of this sequence was not observed. Our assay of repression through heat shock-mediated ectopic expression can be interpreted with confidence only at nc 13 and nc 14, but not earlier (Fig. S3 *A* and *B*), and suggests an inability to assay repression of hb_P2, which emerges early. Collectively, these results show that Run and Su(H) differentially repress *hb*-associated enhancers, and sensitivity to repression for the most part correlates with ChIP-detected occupancy of these factors to enhancer sequences at the endogenous *hb* locus (Fig. 4*C*).

Next, we investigated how these sensitivities to Run and Su(H) at the enhancer level relate to changes in endogenous *hb* expression. Ectopic expression of these factors also affected expression of endogenous *hb*, most clearly at the early stage. At late nc 13, anterior *hb* expression appears shifted anteriorly upon ectopic expression of Run and Su(H), whereas posterior expression of *hb* associated with the hb_stripe enhancer specifically was completely absent, supporting the view that the hb_stripe enhancer is not active yet (i.e., delayed; Fig. 4*H*). However, the effect of ectopic expression of Su(H) on anterior expression of *hb* is stronger than that observed with Run and likely relates to the delay of hb_stripe enhancer as well as hb_shadow in the case of Su(H). Nevertheless, these effects on *hb* expression appeared transient because, by mid-nc 14, *hb* expression is similar to WT (Fig. S4*D*). Heat shock-mediated ectopic expression is only effective starting at nc 13 (Fig. S3*B*). It is possible that ectopic expression at earlier stages would be necessary to support lasting effects on *hb* (and *tll*) expression, but we favor the view that Su(H) and Run regulate the timing of enhancer switching because of the results of mutant analysis.

Transient effects on *hb* expression were also identified in mutants. We found that *hb* expression is turned on earlier in *Su(H)* and *run* mutants, suggesting regulation of this enhancer by these factors (Fig. S4*A*). *hb* is expressed in an expanded domain at the anterior region of *Su(H)* mutants compared with WT, but this expansion appears transient, as, in fully cellularized embryos, the expression in mutants is similar to WT (Fig. S4*B*, Su(H) vs. WT). It is likely that this phenotype relates to prolonged action of the hb_shadow or hb_P2 enhancers, which support expression at the anterior cap. On the contrary, in *run* mutants, a stripe of *hb* expression is observed at the anterior of embryos in early nc 14, stronger in expression than in WT embryos (Fig. S4 *A*–*C*, run vs. WT). This result suggests that the hb_stripe enhancer comes on earlier and is possibly derepressed in *run* mutants.

Run and Su(H) Regulate Patterning Throughout the Embryo, Along the AP Axis as Well as the DV Axis. As these results support the idea that Su(H), in addition to Run, regulates gene expression along the AP axis, we investigated whether, inversely, Run in addition to Su(H) might support DV patterning. *snail* (*sna*) and *short gastrulation* (*sog*) are genes expressed in ventral and lateral regions of *Drosophila* early embryos (3). Previously, we showed that expression of these genes is altered in mutant embryos derived from *Su(H)* germline clone females: the *sna* boundary is unsharp and levels of expression are lower, whereas the *sog* expression domain appears expanded dorsally (7) (Fig. 5*A*). In *run* mutant embryos, *sna* expression domain appears relatively unaffected, but, in contrast, *sog* is expanded relative to WT, but not to the extent observed in *Su(H)* mutants (Fig. 5*A*).

We next assayed whether Run and Su(H) function coordinately to regulate *sog* expression by acting on particular enhancers with the *sog cis*-regulatory system, as observed for *tll* and *hb*. Two enhancers, sog_Intronic and sog_Distal, control *sog* gene expression in the early embryo (20, 21). Ectopic expression of *hs-Su(H)* throughout the embryo leads to complete downregulation of expression from both enhancers, sog_Intronic and sog_Distal; on the contrary, expression of *hs-run* fails to downregulate either (Fig. S5*C*). Repression of both enhancers by

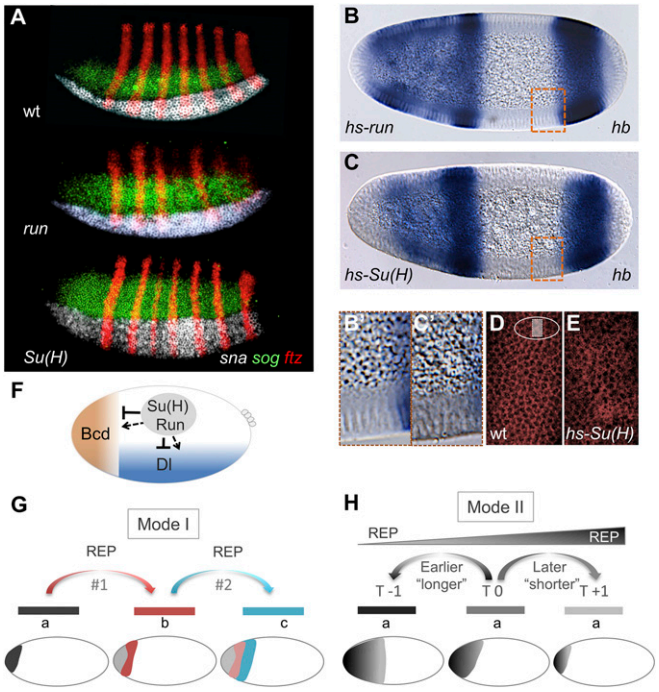

Fig. 5. Ubiquitous repressors regulate enhancer action across embryonic axes. (*A*) FISH using riboprobes to *sna* (white), *sog* (green), and *ftz* (red) show transcript expression domains within WT as well as *run*$^{-}$ and *Su(H)*$^{-}$ mutant embryos (mid-nc 14). (*B* and *C*) Ectopic expression of *Su(H)* through heat shock of *hs-SuH* embryos results in cellularization defects at late cycle 14 (*C* and *C*′). In contrast, no such cellularization phenotypes are observed upon heat shock of *hs-run* (*B* and *B*′) or WT embryos (Fig. S7*C*). (*B*′ and *C*′) Magnified views of *B* and *C*, respectively. (*D* and *E*) Fluorescent staining of embryos shows anomalous distribution of cell membranes within *hs-Su(H)* embryos (*E*) compared with WT (*D*) at mid-nc 14. Embryos in *B*–*E* processed by in situ hybridization using *hb* (*B* and *C*) or *Kr* (*D* and *E*) probes. Although expression of these genes is not necessarily relevant to cellularization defects, this confirms that the embryos are fertilized and development had progressed. (*F*) Broadly acting transcription factors Su(H) and Run encompass multiple roles in patterning by acting as repressors to regulate gene expression along AP and DV axes together with Bicoid (Bcd) and/or Dorsal (Dl) morphogens, respectively. As these factors are known to exhibit dual function, their roles as activators may also be more widespread. (*G* and *H*) Two different mechanisms by which broadly expressed repressors may impact spatiotemporal patterning are depicted. Repressors may regulate the timing of action for different enhancers acting in series (*G*) or, alternatively, repressors may influence the length of time a single enhancer is active (*H*) to impact spatiotemporal outputs.

Su(H) likely explains why expansion of *sog* is observed in *Su(H)* mutant embryos. In contrast, the failure of ectopic Run to repress either enhancer made it unclear why *sog* is expanded in *run* mutants (Fig. 5*A*). However, we found evidence that the sog_Distal enhancer exhibited expanded expression in *run* mutants, and this enhancer sequence also showed binding of Run by ChIP (Fig. S5 *A* and *C*). It is possible that Run's ability to repress DV genes is context-dependent, depending on the binding of other factors to enhancer sequences in tandem on DNA to support Run's activity as a repressor (or activator).

Run and Su(H) are dual-function transcription factors that can function as repressors or activators. The binding of other transcription factors locally to enhancers may cause Run and/or Su(H) factors to locally flip in activity from repressor to activator and vice versa (Fig. 5*F*) (5, 10, 22). It is possible that the spatially localized repression of some enhancers observed in this study (e.g., Fig. 3 *B* and *F*, HC_07, HC_09, and Fig. S2*A*, HC_02) may relate to such context-dependent action of these factors.

To test whether Run and Su(H) apply their repressive effects via direct binding to specific enhancers, we mutated their binding sites to other nucleotides (Fig. S6). Three Su(H)-binding sites within the hb stripe enhancer (Fig. S6*A*) and one Run-binding site within the sog_Distal enhancer were mutated (Fig. S6*B*). Finally, we crossed all hb_stripe and sog_Distal reporter constructs into *hs-run* and *hs-Su(H)* backgrounds (Fig. S6 *C* and *D*), showing that the ability of these factors to repress expression of reporters was dependent on presence of binding sites. Together, these experiments show that the aforementioned transcription factors have direct repressive activity along the two axes.

Ectopic Expression of Su(H) Leads to Defective Cellularization as Exhibited by Zelda Mutants. Surprisingly, we identified shared phenotypes between *zelda* mutants and overexpression of Su(H), as both exhibit cellularization defects (Fig. 5 *C*, *C′*, and *E* and Fig. S7*C*) (23). This phenotype was not associated with ectopic Run expression (Fig. 5 *B*, *B′*, and *D*), suggesting that Su(H) likely supports a distinct and likely wider role in embryonic patterning than Run. In addition, lethality was higher upon ectopic expression of Su(H) compared with Run (Fig. S7), but ectopic Run causes a reduction in male viability, likely because of its previously characterized role in the regulation of the sex-determinant gene *Sex lethal* (24) (Fig. S7). It is possible that Su(H) and Zelda share targets and that Su(H)-mediated repression acts to counterbalance Zelda-mediated activation.

Shared use of key transcription factors, such as Su(H) and Run, across orthogonal axes may help to integrate patterning throughout the embryo and support robust development. Although a standard mechanism of repression involving spatial regulation of activator function is used to establish the posterior boundaries of particular patterns, for instance in the repression of type I patterns by Run (6), our results provide evidence of an additional mechanism of action in which the broadly expressed repressors Run and Su(H) function to fully repress expression of enhancers throughout the embryo to regulate enhancer timing of action and thereby impact spatiotemporal outputs of gene expression. This could be accomplished in at least two ways: regulation of the timing of enhancer initiation for multiple elements acting in series within *cis*-regulatory systems (Fig. 5*G*) or by controlling the length of time that one particular enhancer is active (Fig. 5*H*). A role for broadly expressed repressors in the regulation of enhancer timing is likely a conserved mechanism of action and may extend beyond patterning to temporal regulation of gene expression in general (25).

Materials and Methods

Fly Stocks and Crosses. *yw* was used as WT if not otherwise noted. Su(H)Δ47 FRT40A P[l(2)35Bg +]/CyO (5), run^3/FM7 (Bloomington stock no. 56499), *hs-run* (12), and *hs-Su(H)* (15) fly stocks were used. Details regarding generation of germline clones and heat-shock protocol for ectopic expression are in *SI Materials and Methods*.

Reporter Constructs Analyzed. A total of 14 reporter constructs containing AP enhancers from all three types (0, I, II) of enhancer sequences occupied by Bicoid in vivo were randomly selected from the 2012 study of Chen et al. (6) and assayed. Su(H)- and Run-mutated binding sites of hb_stripe and sog_Distal enhancers were chemically synthesized (GenScript). Mutated site sequences and their WT equivalent fragments are listed in *SI Materials and Methods* (Table S1).

In Situ Hybridizations, Immunohistochemistry, and Image Processing. Embryos were collected, fixed, and stained by using standard conditions (20). Additional information is provided in *SI Materials and Methods*.

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